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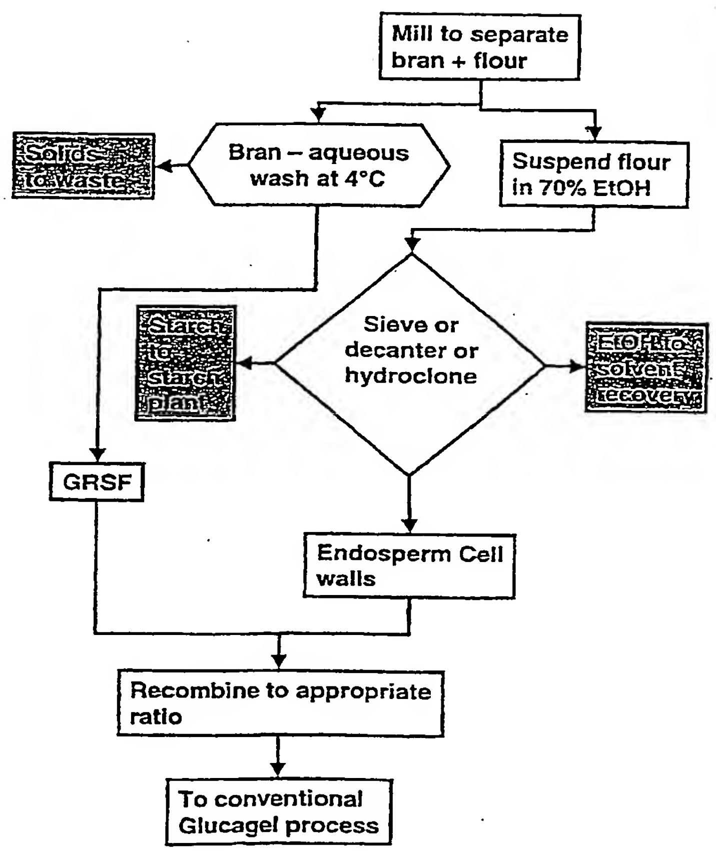
(54) Title: BETA GLUCAN EXTRACTION FROM CEREALS

(57) Abstract

The invention relates to an improved procedure for preparing gelling $\beta$–glucan solutions from cereals. In particular it relates to the protection of a $\beta$–glucan release–stimulating factor or factors that is/are important in the release of gelling $\beta$–glucan from cereal. The release–stimulating factor may be removed from the cereal whilst starch is physically separated or hydrolysed or a release–stimulating factor from another source may be readded after starch separation or hydrolysis.



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# Beta glucan extraction from cereals

## Field of invention

This invention relates to an improved process for the preparation of β-glucan solutions. It also relates to the protection of a factor important in the preparation of an improved β-glucan solution from cereals. The products may be useful as therapeutic agents, biocompatible films and as additives in food, cosmetics, animal feeds, and feedstocks for industrial and biological processing.

## Background of invention

β-Glucan polysaccharides present in cereals are comprised of D-glucopyranosyl units. The units are linked together by (1->3) or (1->4) β-linkages. β-Glucans of this type comprise up to approximately 15% of the solids in oats and barley cereals. They typically have a molecular weight of around 2.5 million.

β-Glucans are useful as soluble dietary fibre. When consumed by humans, soluble fibre remains undigested except by colonic microflora in the lower intestines. This enhances the growth of bacteria beneficial to health. Soluble dietary fibre is believed to have a role in the prevention of certain diseases including colonic cancer and diseases associated with high serum cholesterol levels. Soluble fibre can be used to treat and prevent constipation, to improve bowel regularity, and to regulate the glycaemic response associated with the digestion of many substances.

β-Glucans are considered to have hypocholesterolaemic activity. β-Glucans are also useful as food ingredients. They have neutral flavours and provide bulk in addition to having desirable mouthfeel and texture characteristics. In this context, β-glucans are known as fat replacers in some foods.

The reported methods of extracting β-glucans from cereal involve a number of steps.

First, the milled cereal is treated to deactivate enzymes associated with the cereal. Then the β-glucan is extracted from the cereal into warm water after which the solids are removed from the solution.

Large quantities of water-miscible organic solvents are added to the solution to precipitate the β-glucan, generally along with other polysaccharides. The β-glucan is of low purity and of generally high molecular weight. It is also known to carry out the enzyme deactivation step after the water extraction rather than as a first step. The deactivation step inhibits hydrolysis of the β-glucan thereby maintaining a high average molecular weight of the β-glucan.

Aqueous solutions of the β-glucan obtained via the known methods have minimal functionality with respect to temperature. They are generally high molecular weight gums and form viscous aqueous solutions although they do not redissolve in water easily.

The β-glucans obtained by the reported methods can contain arabinoxylans and starch. These impurities can be responsible for the formation of viscous aqueous solutions during extraction that are difficult to process. Gummy or tacky solids can result when the solids are recovered as a dry product. These products are difficult to redissolve, even at high temperatures.

The known methods for recovering β-glucan from the aqueous solution include precipitation of the β-glucan by the addition of a water miscible organic solvent (such as ethanol) followed by filtration, and spray- or freeze-drying of the precipitate.

PCT/NZ97/00119 describes a process for obtaining β-glucan from cereal including mixing the cereal with water to form a slurry of an aqueous solution of β-glucan and a solid residue such that the β-glucan is partially hydrolyzed by one or more enzymes associated with the cereal, separating the aqueous solution and recovering the β-glucan from the aqueous solution.

PCT/NZ97/00119 also provides a process for recovering β-glucan from an aqueous solution of β-glucan comprising freezing the solution, thawing the solution to give a precipitate in water and separating the precipitate from the water.

Barley flour when mixed with water over a range of temperatures releases β-glucan into the soluble phase. On freezing and thawing, this β-glucan forms a β-glucan gel that can be collected by filtration or centrifugation. This gel is of commercial interest because of its physical properties in foods and because it delivers β-glucan which may have beneficial medical properties.

PCT/NZ97/00119 is incorporated into this specification by reference.

## Object of invention

It is an object of this invention to provide an improved procedure for preparing β-glucan solutions for use in processes for recovering β-glucan from aqueous solutions such as described in PCT/NZ97/00119.

It is a further object of this invention to provide an improved procedure for producing a β-glucan-enriched fraction of a cereal.

It is a further object of this invention to protect a β-glucan gel Release Stimulating Factor to assist the release of β-glucan from a β-glucan-enriched fraction into solution.

## Summary of invention

In a first aspect of this invention there is provided a process for treating cereal such that the treated cereal has an improved capacity to produce gelling β-glucan, comprising:

(a) mixing the cereal with a buffer to form a slurry of an aqueous cereal extract and a solid residue such that the solid residue still contains the majority of the native β-glucan,

(b) separating the aqueous solution containing a factor or factors important in the release of β-glucan from the solid residue,

(c) separating the starch from the solid residue,

(d) adding the aqueous solution containing the factor to the β-glucan–containing residue such that the β-glucan is released into solution.

The factor or factors can be a gelling β-glucan release stimulating factor (GRSF).

The β-glucan solution can be in a form that is capable of forming a gel when the solution is frozen then thawed.

The invention also provides a process for treating cereal such that the treated cereal has an improved capacity to produce gelling β-glucan comprising:

(a) mixing the cereal with water, a buffer or an aqueous solution of a polar organic solvent to form a slurry of an aqueous cereal extract and a solid residue;

(b) separating the aqueous extract containing a factor or factors important in the release of β-glucan;

(c) separating the starch from the solid residue,

(d) adding any β-glucan release-stimulating factor(s) in buffer to the solid residue such that β-glucan is released into solution.

The said any β-glucan release-stimulating factor(s) added to the solid residue may be other than that produced by step (b).

The buffer used may be water, or may be water with the addition of ionic materials to form the buffer with a salt concentration between 0 molar and 5 molar, preferably less than 1 molar, more preferably 100 mM.

The ionic materials may be any salts capable of forming a solution up to 5 molar between pH2 and pH12, preferably between pH3.5 and pH10, more preferably between pH5 and pH8. The preferred salts are potassium dihydrogen phosphate and potassium hydroxide in mixtures defined by the desired pH. The preferred ratio of buffer to cereal is between 1 part of cereal to 1 part of buffer and 1 part of cereal and 50 parts of buffer, more preferably between 5 and 20 parts of buffer to 1 part of cereal.

The preferred temperature for the separation of GRSF and starch is between 0 °C and 70 °C, more preferred between 1 °C and 25 °C, yet more preferred between 2 °C and 10 °C. The preferred temperature for releasing the β-glucan from the residue is as specified in PCT/NZ97/00119.

Also provided is a process for preparing a β-glucan-enriched fraction of a cereal grain, comprising:

- separating the starch from the whole ground cereal or drymilled fractions thereof by washing with an aqueous solution of a polar organic solvent such that the washing also removes components soluble in such solution, but not β-glucan, and such that the composition of the washing solution does not affect the activity of a desirable enzyme system that is stimulated by a β-glucan release stimulating factor.

The polar organic solvent can be any polar organic solvent. The preferred solvent should be capable of forming a mixture with water such that the resultant mixture does not dehydrate or defat cereal cell walls sufficiently to remove the enzyme activity necessary for release of a gelling β-glucan. The preferred polar solvent is

ethanol, and the preferred mixture of ethanol to water is from 10% to 80%, more preferred is 30% to 80%, yet more preferred is 70% ethanol and 30% water. The preferred temperature for the washing process is between −20 °C and 60 °C, more preferably between −20 °C and 20 °C.

The β-glucan may be recovered from the β-glucan-enriched fraction in accordance with PCT/NZ97/00119.

The preferred ratio of β-glucan-containing residue or β-glucan-enriched fraction to GRSF-containing aqueous solution is such that the calculated ratio of original cereals from which the components of the mixture are derived lies between 1:10 and 10:1. More preferably, this lies between 1:5 and 2:1 for the ratio (solution source)/(residue or enriched fraction source)

The preferred temperature for this procedure lies between 4 °C and 80 °C, more preferably between 30 °C and 70 °C, yet more preferably between 50 °C – 60 °C.

In accordance with another aspect of this invention, there is provided a process for preparing a gelling β-glucan Release Stimulating Factor (GRSF) solution, comprising:

- milling whole cereal grain,
- mixing the resultant whole flour or a fraction thereof to a slurry with a buffer or aqueous organic solvent such that minimum β-glucan release occurs,
- separating the aqueous solution from the solid residue, wherein the aqueous solution contains the GRSF,
- removing the organic solvent (if used) by known means, such that the stimulating activity is retained.

The GRSF activity may optionally be concentrated from the aqueous solution.

Low-molecular weight solutes may be removed from the aqueous solution.

The buffer used may be water, or may be water with the addition of ionic materials to form a buffer with a salt concentration between 0 molar and 5 molar, more preferably less than 1 molar, yet more preferably 100mM.

The ionic materials may be any salts capable of forming a solution up to 5 molar between pH2 and pH12, preferably between pH3.5 and pH10, more preferably between pH5 and pH8. The preferred salts are potassium dihydrogen phosphate and potassium hydroxide in mixtures defined by the desired pH. The preferred ratio of buffer to cereal is between 1 part of cereal to 1 part of buffer and 1 part of cereal and 50 parts of buffer, more preferably between 5 and 20 parts of buffer to 1 part of cereal.

The polar organic solvent can be any polar organic solvent. The preferred solvent should be capable of forming a mixture with water such that the resultant mixture does not dehydrate or defat cereal cell walls sufficiently to remove the enzyme activity necessary for release of a gelling β-glucan. The preferred polar solvent is ethanol, and the preferred mixture of ethanol to water is from 80% to 10%, more preferred is 80% to 30%, yet more preferred is 70% ethanol and 30% water. The preferred temperature for the washing process is between −20 °C and 60 °C, more preferably between −20 °C and 20 °C.

Non-essential enzyme activity may optionally be reduced by known physical processes.

The GRSF may optionally be dried using known processes.

The cereal used in this invention may be any β-glucan-containing grain or plant material including, but not limited to, barley, oats, rice, rye, triticale, maize and wheat. The preferred cereal is barley.

The cereal may be any grain, including, but not limited to barley, oats, wheat, maize or rice. The preferred cereal is barley. Further, the cereal may be any grain comminuted in any fashion, or any component or millstream from such comminution. The preferred cereal is pollard or bran from barley.

The invention provides a release stimulating factor preparation and its reservation while starch and cell walls are separated. The invention also provides the use of a non-dehydrating, non-defatting solvent that will not dissolve β-glucan. The invention results in being able to produce enriched raw material for gelling β-glucan production so that the cost of the freeze-thaw component of the process may be reduced, and the ability to produce an extraction system with reduced extraneous enzyme activity so that the molecular weight of the gelling β-glucan produced can be maintained at a higher level if desired.

These and other advantages will be apparent to those skilled in the art.

Waxy cereals have the advantage over non-waxy cereals that less leaching of amylose from the starch granules into the extraction solution occurs.

The invention results in a process for producing β-glucan which controls molecular weight, is cheaper than known methods, results in a higher degree of purity of β-glucan and is better able to exert control over microbial contamination.

## Brief Description of Drawings

Embodiments of the invention are now described, by way of example only, with reference to the drawings in which:

Figure 1 shows the effects of silver nitrate and heat on whole flour β-glucan gel production;

Figure 2 shows the effects of adding β-glucan to flour;

Figure 3 shows the effects of removing the soluble fraction;

Figure 4 shows the effects of addition of soluble fraction to cell walls;

Figure 5 shows the effects of adding supernatant to cell walls;

Figure 6 shows the fractionation of soluble fraction by filtration;

Figure 7 shows the effects of heat or low pH treatment on soluble fraction;

Figure 8 shows that the extended incubation of cell walls with GRSF preparations does not affect the gelling ability of gelling β-glucan;

Figure 9 shows the effects of buffer and pH;

Figure 10 shows the effect of boiling the cell wall fraction;

Figures 11a and 11b are a comparison of gelling β-glucan production from water-washed and ethanol-washed cells. (a) water-washed (b) ethanol-washed. Treatment 1, 2 ml cell walls plus 5 ml water; treatment 2, 2 ml cell walls plus 2 ml supernatant; treatment 3, 2 ml cell walls plus 3 ml supernatant; treatment 4, 2 ml cell walls plus 4 ml supernatant; treatment 5, 2 ml cell walls plus 5 ml supernatant; treatment 6, 2 ml cell walls plus 2 ml supernatant plus 3 ml water; treatment 7, 2 ml cell walls plus 3 ml supernatant plus 2 ml water; treatment 8, 2 ml cell walls plus 4 ml supernatant plus 1 ml water; treatment 9, 2 ml cell walls plus 5 ml supernatant. (Treatments 5 and 9 are essentially replicates.);

Figure 12 shows the effects of varying the soluble fraction concentration;

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**Figure 13** shows the effects of varying the amount of soluble fraction;

**Figure 14** shows the effects of various salts on gelling β-glucan formation;

**Figure 15** shows the total gelling β-glucan stimulating ability of supernatants of flour fractions;

**Figure 16** shows the effect of heat treatment on gelling β-glucan production. The top figure shows the effect of heat treatment of cell walls assayed with water (odd numbers) or non-heat-treated supernatant. Heat treatments were 20 °C (1 & 2), 50 °C (3 & 4) 65 °C (5 & 6), 80 °C (7 & 8) 95 °C (9 & 10);
The lower figure shows the effect of heat treatment on GRSF extracts in combination with untreated cell walls. The data are in sets of three (1 ml extract, 3 ml extract, 5 ml extract). Untreated extract (a,b,c), 50 °C (d,e,f) 65 °C (g,h,i) 80 °C (j,k,l) 95 °C (m,n,o). p: no cell walls and no heat treatment;

**Figure 17** shows gelling β-glucan yield from water and ethanol washed cell walls prepared from flour of barley breeders line 1831.1;

**Figure 18** shows GRSF thermostability; and

**Figure 19** is a schematic diagram of the improved procedure for producing a solution with an improved capacity to produce gelling β-glucan.

**Detailed Description of the Invention**

This research investigates the biochemical mechanisms behind the release and gelation properties of β-glucan in barley. (GG = gelling β-glucan)

Example 1 - Preliminary research - establishing a small scale assay

The standard method for assaying gelling β-glucan production involves the following steps:

Mix 5g barley flour with 25ml water
Incubate at 50°C for 2 hours
Centrifuge to remove solids
Freeze liquid at -15°C for 12 hours
Thaw at room temperature
Collect gel by filtration
Wash 2x with distilled water and 1x with ethanol
Dry in an oven at 80°C
Weigh

Development of the 1 g method

A small-scale method for GG production and analysis was required in order to process larger numbers of samples and to facilitate purification of active components. The following assay was established:

Weigh 1 g of flour into a 15 ml Falcon tube
Add 5 ml water
Mix well
Incubate 2 hours at 50°C
Centrifuge at 4500 rpm in spin-out rotor for 10 min at room temperature
Decant supernatant to a fresh tube weighed to 1 mg
Freeze at -15°C overnight
Thaw at room temperature
Centrifuge at 4500 rpm in spin-out rotor for 10 min at room temperature
Carefully discard supernatant - spin tubes for 30 sec to collect remaining supernatant, remove with a Pasteur pipette
Weigh tube and calculate wet weight of GG.

(Note that this assay gives the wet weight of GG produced - not the dry weight as above. However, wet weight was shown to be highly correlated with dry weight across a sample set).

Example 2 - Whole flour studies

In each of the experiments described in this section, the flour used was from a roller-milled sample of a breeding line coded 1795.

(a) Effects of silver nitrate and heat

Initial studies were conducted using the 1 g whole flour assay in order to investigate the sensitivity of gelling β-glucan production to inhibition by silver nitrate or heat treatments that frequently eliminate enzyme activity (see Fig. 1). Silver nitrate was added to the water/flour mixture to the concentrations indicated immediately after forming the water/flour mixture and before incubation at 50°C. The heat treatments indicated refer to heating of the flour prior to the addition of water.

Clearly in this assay system, silver nitrate was a poor inhibitor of β-glucan gel formation. This may be the result of either (a) insensitivity of enzymes involved, or (b) restricted access of silver nitrate to the enzymes. Dry heat was also shown to have limited effect. While dry heat might be expected to have a much less denaturing effect than heat in the presence of excess water, this result suggests that gelling β-glucan formation is relatively insensitive to heat.

(b) Effect of adding β-glucan to flour

Standard β-glucan from either barley or oats (Megazyme Ltd, Bray, Eire) was added to the standard 1 g flour assay prior to the addition of water. No stimulation of gelling β-glucan wet weight formation was obtained (Fig. 2).

(c) Effect of removing the soluble fraction of the flour by washing or dialysis

Flour was treated in two ways: firstly, by dialysis (nominal 12kD molecular weight cut-off) overnight against 50 mM Sodium acetate buffer (pH 5), and secondly, by washing three times with water to remove soluble material. Water and buffer controls are included. Clearly the removal of the soluble fraction by either method significantly reduces the amount of gelling β-glucan formed (Fig. 3).

Conclusions

The experiments described in (a), (b) and (c) demonstrate limited progress can be made through the analysis of whole flour fractions.

- The effect of heat and inhibitors is difficult to study in the presence of large amounts of starch and protein.
- The gelling β-glucan formation system is not simply stimulated by adding more purified β-glucan and therefore the system should contain "native" β-glucan.
- Experiment (c) demonstrates that a soluble fraction is required for maximum gelling β-glucan formation.

Example 3 - Separation and analysis of flour components

A simple flour fractionation procedure was developed as follows:

Add 125 ml of water to 25 g of flour and mix well over 15 minutes;
Centrifuge at 10,000 rpm for 10 min at 4°C;
Remove supernatant and store at 4°C - designated the Soluble Fraction;
Transfer pellet to a filter cloth (preferably a 70 micron nylon filter, but a porous cloth will suffice);
Wash with tap water with agitation until the wash water runs clear (approximately 20 minutes);
Transfer retained material to a tube; and

Allow to settle and decant off excess water

The settled material is designated the Cell Wall Fraction

(a) Effect of mixing the cell wall and soluble fractions

In this experiment, 2 ml of settled cell walls were transferred to a 15 ml Falcon tube. Either 5 ml of water or soluble fraction was added and the 7 ml reaction mixture incubated at 50°C and processed as described for the 1 g flour assay. Figure 4 shows the effect of adding soluble fraction to the cell wall fraction. The sample labelled soluble fraction contained 5 ml of supernatant, no cell wall fraction, and an additional 2 ml of water.

The experiment demonstrates that gelling β-glucan formation from barley cell walls is stimulated by the soluble fraction. The experiment was repeated and the repeat experiment is shown in Figure 5.

Conclusion

- Gelling β-glucan production by either the cell wall or soluble fractions alone is substantially lower than gelling β-glucan production when the two components are mixed.
- This result confirms the earlier observation that there is a factor in the soluble fraction that acts on cell walls, inducing gelling β-glucan formation.

(b) Experiments on the nature of the soluble component

Filtration: In this experiment 25 ml of soluble fraction (prepared as described above) was filtered in an Amicon ultrafiltration device fitted with a 10,000 MW cut-off membrane. 20 ml of filtrate were obtained and 4 ml of retained material. Assays were conducted in duplicate using either 1 ml or 4 ml of the filtrate, or 0.5 or 1.5 ml of the

retentate, made up to 4 ml with water. The assays included 2 ml of Cell Wall fraction. The results are shown in Figure 6. Controls with 2 ml of cell wall fraction with either water or unfiltered soluble fraction are included.

Conclusion

- The low molecular weight filtrate contained no gelling β-glucan release stimulating activity.
- The assays containing high molecular weight retentate retained the ability to form gelling β-glucan. In combination with the results of the dialysis experiment (Fig. 3), the results suggest that the gelling β-glucan release-stimulating activity is a small macromolecule between 10,000 Dalton and 12,000 Dalton apparent molecular weight.

Heat and pH treatments: Figure 7 shows the effect of heat or low pH treatments on the soluble fraction's ability to stimulate gelling β-glucan formation. A water control and controls with 1 ml, 3 ml or 4 ml of supernatant added (plus water to 4 ml total volume) are included.

Soluble fraction was prepared using the standard procedure. The heat-treated samples were boiled for 10 minutes, centrifuged and the supernatant added into the assay. The low pH samples were adjusted to pH 3.5 with acetic acid and held for 10 minutes before returning the sample to pH 5.5 with NaOH.

There is some effect of each treatment on the amount of gelling β-glucan formed.

Conclusion

This experiment suggests that the soluble factor that stimulates gelling β-glucan formation is stable to treatments normally expected to eliminate, or substantially reduce, enzymatic activity. Note that this experiment is complicated by the (probable) presence of soluble β-glucan in the soluble fraction. It would be preferable to do this experiment with M737 (a very low β-glucan barley mutant) supernatant, since M737

produces soluble fractions almost entirely incapable of producing gelling β-glucan when assayed in the absence of cell walls. However, it has been shown that boiling before freezing prevents solubilised β-glucan forming gelling β-glucan. Therefore, we would not expect the solubilised β-glucan to be contributing to the stimulating activity seen here. In fact, the results of this boiling experiment are consistent with the loss of the contribution of the soluble β-glucan and the near full retention of the gelling β-glucan gel release stimulating activity.

Time course experiment

Washed cell walls were combined with 5 ml of supernatant per 2 ml of settled washed cell walls. Samples were taken at various times after initiation of the 50°C incubation, centrifuged immediately, and frozen overnight. Figure 8 shows the result of this experiment.

Conclusion

- This result is consistent with previous results using whole flour systems.

Effect of buffer and pH on gelling β-glucan formation

β-Glucan gel formation was assayed at different pHs using different buffers. Washed cell walls and soluble fraction were prepared using the standard method. Buffer was added to the reaction mixtures prior to the 50°C incubation. The final buffer concentrations and pHs used were:

- 50 mM Sodium acetate buffer pH 5.0
- 50 mM Sodium phosphate buffer pH 7.0
- 50 mM Tris-HCl buffer pH 9.0

Assays were conducted with soluble fraction or with water. The results are shown in Figure 9.

Conclusion

– The trend is towards lower pH values giving higher yields of β-glucan gel.

Note

A more comprehensive survey of buffer types and pH may provide further optimisation of the gelling β-glucan production process and this may give commercially useful improvements in the yield of gelling β-glucan.

Inhibition of gelling β-glucan formation by boiling the cell wall fraction:

The production of gelling β-glucan from cell wall fractions in:

- 5 ml water
- 5 ml supernatant

was compared to gelling β-glucan production in water following boiling the cell walls for 5 min prior to the 50°C incubation.

Conclusion

Production of gelling β-glucan from the cell wall fraction in the absence of soluble fraction is reduced substantially by boiling for 5 min.

Ethanol-washed cell walls vs water-washed cell walls

Cell wall fractions were prepared as follows

Water washed:

25 g flour and 100 ml water were mixed and stirred for 15 min at 4°C, then centrifuged for 10 min at 10 k rpm at 4°C. – The supernatant was reserved for use in

the final assay, and the pellet was transferred to a 70-micron screen and washed exhaustively with water to remove starch.

Ethanol washed:

20 g flour was washed with 70% ethanol by placing flour in a bag made of 70-micron nylon bolting. The bag was placed inside a 1 litre bottle and washed 5 times with 200 ml of 70% ethanol to remove starch – the wash was thoroughly equilibrated with the bag contents each time before removing. Cell walls were quantitatively transferred to a centrifuge tube and water added to 12 ml. The tube was centrifuged at 2k rpm for 30 seconds to pack cell walls and the supernatant discarded.

For both water-washed and ethanol-washed cells, walls were collected by centrifugation and suspended in 45 ml of water. 3 ml aliquots were transferred to each of 15 tubes and centrifuged at 2k rpm for 30 sec. Supernatants were then removed, and the gelling β-glucan production potential assayed according to the treatments in the caption to Figure 11, in which results are given.

<u>Conclusion</u>

Gelling β-glucan production from both preparations was essentially similar. This is consistent with the results of varying the ratio of soluble fraction to cell walls shown below.

Limitations

(1) no β-glucan measurements were made so results are not normalised against original cell wall β-glucan content.

<u>Production of freeze-dried cell walls</u>

Cell walls were prepared from 1795 by the standard water washing procedure and then freeze dried over several days. The experiments described in Figures 12, 13 and 14 used 0.5 g of freeze dried cell walls and various amounts of supernatant or liquid as indicated.

Varying the ratio of soluble fraction to cell wall material

In this experiment, the amount and concentration of Soluble Fraction was varied while cell wall amount was kept constant.

In Figure 12, the effect of varying the concentration of soluble fraction was assayed. All assays contained 0.5 g of freeze-dried cell walls and 10 ml of soluble fraction or water in different proportions, as indicated in the figure.

Conclusion

Increasing the concentration of soluble fraction above 40% had little effect in this set of experiments. This experiment is essentially a repeat of the experiments described in Figure 4 and Figure 5, except using freeze-dried cell walls. The results of this experiment are more similar to the results in Figure 4 than Figure 5.

In Figure 13, the effect of adding different volumes of soluble fraction to a fixed amount (0.5 g) of freeze dried cell wall is shown.

Note that 0.5 g of freeze dried cell walls is equivalent to about 2 ml of hydrated cell walls.

It was noted during the course of this experiment that after freeze/thaw, the entire volume of the 2 ml sample was occupied by a loose gel. On centrifugation this gel was compacted.

Conclusion

- The addition of further soluble fraction above 4 ml did not further stimulate the formation of gelling β-glucan, but rather, led to a decline in production. This indicates that there was sufficient stimulating factor in 4 ml of soluble fraction to give full stimulation of gelling β-glucan formation. This experiment is therefore fully consistent with results presented in Figures 4,11 and 12, which

suggest that gelling β-glucan formation approaches full saturation when the proportion of soluble fraction is about 40% of the original concentration.

- The addition of further soluble fraction above 4 ml may reduce gelling β-glucan formation by a dilution effect that inhibits gel formation, rather than affecting the release of β-glucan from the cell walls. The soluble fraction may also contain either endogenous or exogenous β-glucanases or cellulases that damage the ability of released β-glucan to gel.

Effect of various salts on gelling β-glucan formation

These assays contained 2 ml of cell walls and 5 ml of supernatant, and various compounds as indicated in the legend to Figure 13.

Conclusion

- None of the salts further stimulated gelling β-glucan formation when added to the incubation mixture.

Example 4 - Distribution of GRSF among milling fractions

Aim: To define the ability of the supernatants from various milling fractions to stimulate β-glucan formation from a constant amount and type of cell walls.

Substrate

A naked, waxy barley breeding line coded 1831.1 was milled using a Buhler roller mill.

A composite barley flour was made by weighing out the following fractions:

| | |
|---|---|
| Break flour | 17.7 g |
| Reduction flour | 39.3 g |
| Pollard | 39.3 g |
| Pollard Flour | 21.3 g |

| | |
|---|---|
| Bran | 24.9 g |
| Bran flour | 7.5 g |
| Total | 150 g |

10 g of the flour was reserved.

140 g was mixed to a slurry with 560 ml of 20 mM KPi buffer at pH 6.0 and centrifuged at 5 k rpm, for 10 min, at 4°C

The supernatant was reserved and the pellet was washed extensively with water through a 70-micron filter

Cell walls were allowed to settle in water, and excess water was removed.

Soluble Fractions

15 g each of break flour, reduction flour, pollard, pollard flour, bran, and bran flour were individually mixed with 60 ml of KPi buffer at pH 6.0.

The samples were centrifuged at 10K rpm, for 10 min, at 4°C. The supernatants were decanted and stored at 4°C.

Assays

2 ml of composite cell walls were placed in each tube, with a total of 6 ml buffer or supernatant. For each supernatant preparation, 1.5 ml supernatant plus 4.5 ml buffer, 3 ml supernatant plus 3 ml buffer, and 6 ml supernatant treatments were included.

Incubation was at 50°C for 2 hours.

Figure 15 shows the results of an experiment that measured the total gelling β-glucan release stimulating ability of the supernatants of the flour fractions. The results represent the sum of β-glucan release from the stimulation of the cell walls and the

addition of the soluble β-glucan from the supernatant. Supernatants from the four flour fractions have little ability to stimulate β-glucan gel formation from cell walls compared to the bran and pollard fractions.

Example 5 - Effect of heat treatment on gelling β-glucan production

Aim: To define the ability of the supernatant and cell wall fractions to generate gelling β-glucan after heat treatments to defined temperatures.

Substrate

A composite barley flour was made by weighing out the following fractions:

| | |
|---|---|
| Break flour | 118 g |
| Reduction flour | 262 g |
| Pollard | 262 g |
| Pollard Flour | 142 g |
| Bran | 249 g |
| Bran flour | 75 g |
| Total | 1000 g |

50 g of the flour was reserved.

The remaining flour was mixed to a slurry with water and then washed through a 70-micron filter until no further starch could be eluted. The cell walls were frozen, and then freeze-dried.

Soluble fraction

50 g of pollard was mixed with 250 ml of KPi buffer (pH 5.8) and allowed to incubate at room temperature for 15 min. The supernatant was collected by centrifuging at 10k rpm for 10 min at 4°C.

Heat Treatment

Heat treatments of room temperature (20°C), 50°C, 65°C, 80°C and 95°C were used. A cell wall/buffer slurry was prepared by mixing 15 g of freeze-dried walls with 120 ml of KPi buffer (pH 5.8). The mixture was stirred well and 2 ml aliquots dispensed into 50 tubes.

Tubes were heat-treated for 15 min.

20 ml aliquots of supernatant were heat-treated in glass vials in a water bath for 15 min. The supernatants were centrifuged for 10 min at 10 k rpm at 4°C to remove precipitated protein.

Sample (p) demonstrates that a large proportion of the total gel is derived from β-glucan solubilised from the pollard fraction. In the upper figure, the odd-number samples show that there is a deterioration in the base level of β-glucan gel production from washed cell walls with increases in temperature. Heat has little effect on the reconstituted cell wall-soluble β-glucan-GRSF system.

The lower figure confirms this result.

Conclusion

– the system has high heat stability.

Limitation

– the data does not give information on the heat stability of GRSF alone – but rather on GRSF plus soluble β-glucan.

<u>Example 6 - GRSF Thermostability</u>

100 ml of GRSF extract was prepared from each of M737 and 1831.1 and placed in a 55°C water bath.

At 0,2,4,7 and 24 hours, 20 ml were withdrawn and used to slurry 4 g ethanol-washed cell walls

A 1 h gelling β-glucan preparation was carried out and β-glucan gel measured.

Gel weight increased with time of extract incubation until 4 hours then declined to hour 7 (remaining stable thereafter with1831.1 (Fig. 18))

Implication:

GRSF was processed after release by a soluble enzyme. This initially increases release stimulating ability then over-processes the GRSF. This suggests that the unknown factor is a protease.

Example 7 - Dialysis experiment

A conventional gelling β-glucan preparation was carried out using 5g flour from 1831.1 and 25 ml of KPi buffer (pH5.8). The supernatant from the freeze-thaw process was retained and used for a second gelling β-glucan extraction, this time using ethanol-extracted cell walls from 1831. The supernatant was ultrafiltered as for example 3.

Ultrafiltration experiment (Ethanol-washed cell walls)

| Treatment | Yield | Mean |
|---|---|---|
| + 5 ml ultrafiltered supernatant | 0.463 | 0.487 |
| | 0.511 | |
| + 5 ml $H_2O$ | 0.596 | 0.6635 |
| | 0.731 | |
| + 5 ml boiled ultrafiltered supernatant | 0.576 | 0.6165 |
| | 0.657 | |

The implication of these data is that no GRSF is available for stimulating a second batch of gelling β-glucan production, but that the supernatant contains thermolabile enzyme activity capable of damaging the gelling capability of the gelling β-glucan released by the endogenous GRSF in the cell wall preparations.

Example 8 -Comparison of β-glucan gel release from water and ethanol-washed cell walls

Supernatants were prepared from three barleys (M737, Himalaya 1002 and Himalaya 1003) known to produce GRSF extracts with very low endogenous gelling β-glucan production, and from Hiproly, GRSF extracts from which produce some endogenous gelling β-glucan. 4 ml of supernatant was added to 2 ml of a slurry of 1 part water to five parts of either water-washed or ethanol-washed cell walls and incubated at 50°C for 2 hours. Results are given in Figure 17.

Implication: Ethanol washing of cell walls produces material that contains approximately 2.5 times more gelling β-glucan precursor compared to water-washed material from the same source.

It is to be understood that the scope of the invention is not limited to the described embodiment and therefore that numerous variations and modifications may be made to these embodiments without departing from the scope of the invention as set out in this specification.

**Industrial applicability**

The invention provides an improved procedure for the release of β-glucan from cereals. β-Glucans are useful as food ingredients. They are known to have hypocholesterolemic activity and to provide bulk as well as desirable texture characteristics in foods and other products.

Claims:

1. A process for treating cereal such that the treated cereal has an improved capacity to produce gelling β-glucan, comprising:

(a) mixing the cereal with water or a buffer to form a slurry of an aqueous cereal extract and a solid residue such that the solid residue still contains the majority of the native β-glucan;
(b) separating the aqueous solution containing a factor or factors important in the release of β-glucan from the solid residue;
(c) separating the starch from the solid residue; and
(d) adding the aqueous solution containing the factor or factors produced to the β-glucan-containing residue such that the β-glucan is released into solution.

2. A process according to claim 1 in which steps (a), (b) and (c) are carried out at between 0°C and 70°C, preferably between1°C and 25°C, yet more preferably 2-10°C and step (d) is carried out at 25-70°C, preferably 50-60°C.

3. A process for treating cereal such that the treated cereal has an improved capacity to produce gelling β-glucan comprising:

(a) mixing the cereal with water, a buffer or an aqueous solution of a polar organic solvent to form a slurry of an aqueous cereal extract and a solid residue;
(b) separating the aqueous extract containing a factor or factors important in the release of β-glucan from the solid residue;
(c) separating the starch from the solid residue; and
(d) adding any β-glucan release-stimulating factor(s) to the solid residue such that β-glucan is released into solution.

4. A process according to claim 3 wherein steps (a), (b) and (c) are carried out at between 0°C and 70°C, preferably between 1°C and 25°C, yet more preferably 2°C -10°C and step (d) is carried out at 25°C -70°C, preferably between 50°C -60°C.

5. A process according to claim 3 in which the polar organic solvent is capable of forming a mixture with water such that the resultant mixture does not dehydrate or defat cereal cell walls sufficiently to remove the enzyme activity necessary for release of a gelling β-glucan.

6. A process according to claim 5 wherein the polar solvent is ethanol.

7. A process according to claim 6 wherein the proportion of ethanol to water is from 80% to 10%, preferably 80% to 30%, more preferably 70% ethanol, 30% water.

8. A process according to any preceding claim in which ionic materials are added to the water to form a buffer with a salt concentration between 0 and 5 molar.

9. A process according to claim 8 wherein the concentration is less than 1 molar.

10. A process according to claim 9 in which the concentration is 100 mM.

11. A process according to any one of claims 8-10 in which the pH of the solution is between pH2 and pH12.

12. A process according to claim 11 in which the pH is between 3.5 and 10.

13. A process according to claim 12 in which the pH is between 5 and 8.

14. A process according to any one of claims 8-13 in which the salts are potassium dihydrogen phosphate and potassium hydroxide.

15. A process according to any one of claims 5-14 in which the ratio of buffer or aqueous organic solvent to cereal is between 1 part cereal to 1 part buffer or aqueous organic solvent and 1 part cereal to 50 parts buffer or aqueous organic solvent, preferably between 5 and 20 parts buffer or aqueous organic solvent to 1 part cereal.

16. A process for preparing a β-glucan-enriched fraction of a cereal grain, comprising:

- separating the starch from the whole ground cereal or drymilled fractions thereof by washing with an aqueous solution of a polar organic solvent such that the washing also removes components soluble in such solution, but not β-glucan, and such that the composition of the washing solution does not affect the activity of a desirable enzyme system that is stimulated by a β-glucan release stimulating factor.

17. A process according to claim 16 in which the solvent is capable of forming a mixture with water such that the resultant mixture does not dehydrate or defat cereal cell walls sufficiently to remove the enzyme activity necessary for release of a gelling β-glucan.

18. A process according to claim 16 or claim 17 wherein the polar solvent is ethanol.

19. A process according to claim 18 wherein the proportion of ethanol to water is from 80% to 10%, preferably 80% to 30%, more preferably 70% ethanol, 30% water.

20. A process according to any one of claims 16-19 where the temperature for the washing process is between -20°C and 60°C, preferably -20°C and 20°C

21. A process for the release of β-glucan into solution according to any preceding claim where the ratio of disabled cereal or β-glucan-enriched fraction to aqueous solution is such that the calculated ratio of original cereals from which the components of the mixture are derived lies between 1:10 and 10:1, preferably between 1:5 and 2:1 for the ratio (solution source)/ (cereal or enriched fraction source).

22. A process according to claim 21 where the temperature for the procedure is between 4°C and 80°C, preferably 30°C -70°C, more preferably 50°C -60°C.

23. A process for preparing a gelling β-glucan Release Stimulating Factor (GRSF) solution, comprising:

- milling whole cereal grain,
- mixing the resultant whole flour or a fraction thereof to a slurry with water or a buffer or an aqueous solution of an organic solvent such that minimum β-glucan release occurs,
- separating the aqueous solution from the solid residue, wherein the aqueous solution contains the GRSF.

24. A process according to claim 23 in which the buffer used is water.

25. A process according to claim 24 in which ionic material is added to the water to form a buffer with a salt concentration of between 0 and 5 molar.

26. A process according to claim 25 in which the concentration is less than 1 molar.

27. A process according to claim 26 in which the concentration is 100 mM.

28. A process according to any one of claims 25-27 in which the pH of the solution is between pH2 and pH12, preferably between pH3.5 and pH10, more preferably between pH5 and pH8.

29. A process according to any one of claims 25-28 in which the salts are potassium dihydrogen phosphate and potassium hydroxide.

30. A process according to claim 23 in which the solvent is capable of forming a mixture with water such that the resultant mixture does not dehydrate or defat cereal cell walls sufficiently to remove the enzyme activity necessary for release of a gelling β-glucan.

31. A process according to claim 23 or claim 30 wherein the polar solvent is ethanol.

32. A process according to claim 31 wherein the proportion of ethanol to water is from 80% to 10%, preferably 80% to 30%, more preferably 70% ethanol, 30% water.

33. A process according to any one of claims 23, 30, 31 or 32 where the temperature for the washing process is between -20°C and 60°C, preferably -20°C and 20°C.

34. A process according to any one of claims 25-33 in which the ratio of buffer to cereal is between 1 part cereal to 1 part buffer and 1 part cereal to 50 parts buffer, preferably between 5 and 20 parts buffer to 1 part cereal.

35. A process according to claim 34 in which, when an aqueous solution of an organic solvent is used, the organic solvent is removed.

36. A process according to claim 35 in which removal is by low temperature evaporation.

37. A process according to any one of claims 26-36 in which the GRSF is concentrated from the aqueous solution.

38. A process according to any one of claims 26-37 where low-molecular weight solutes are removed from the aqueous solution.

39. A process according to any one of claims 26-38 wherein non-essential enzyme activity is reduced.

40. A process according to any one of claims 26-39 wherein the GRSF is dried.

41. A process according to any preceding claim where the cereal is barley.

42. A process according to claim 41 in which the cereal is pollard or bran from barley.

43. A β-glucan enriched fraction produced by the process of any preceding claim.

Figure 1

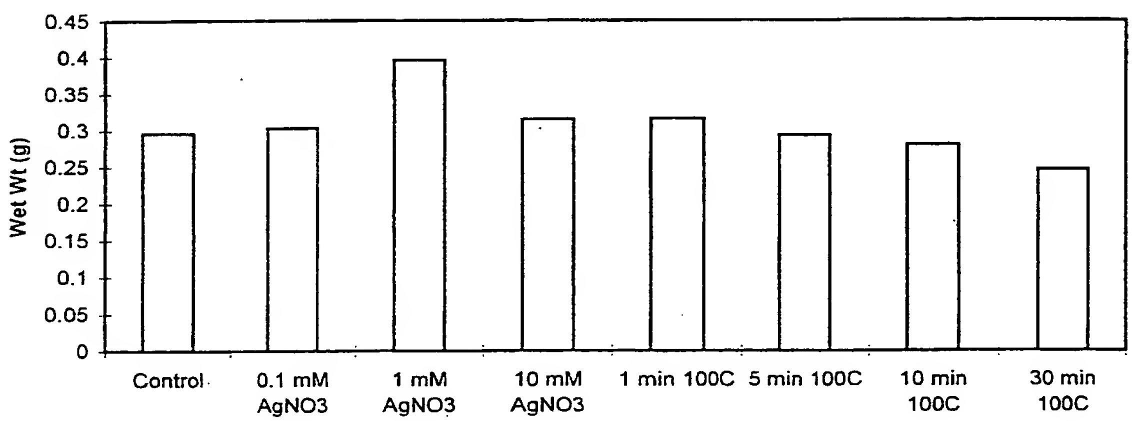



Figure 2

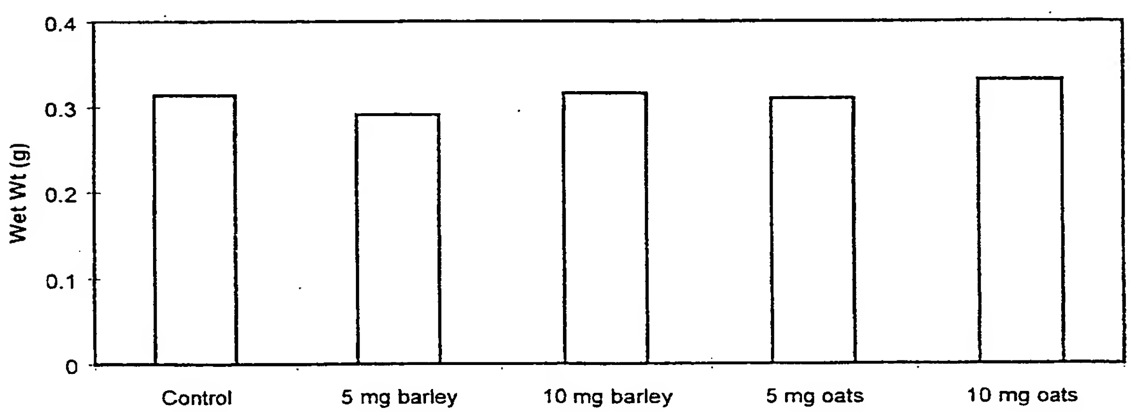


Figure 3

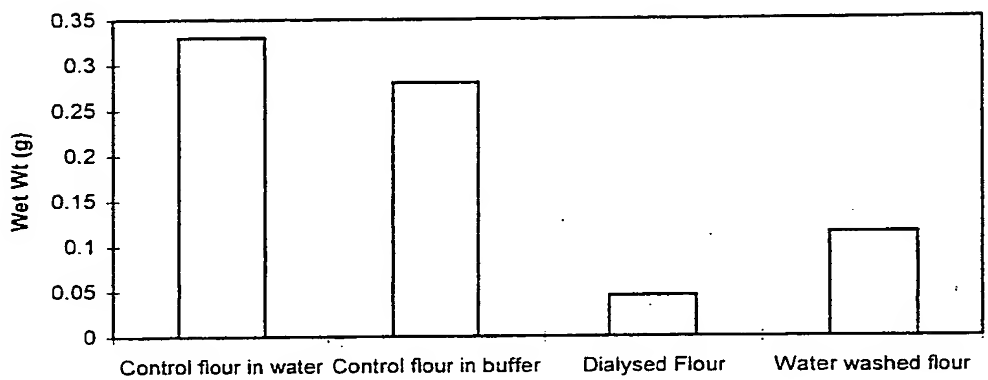



Figure 4

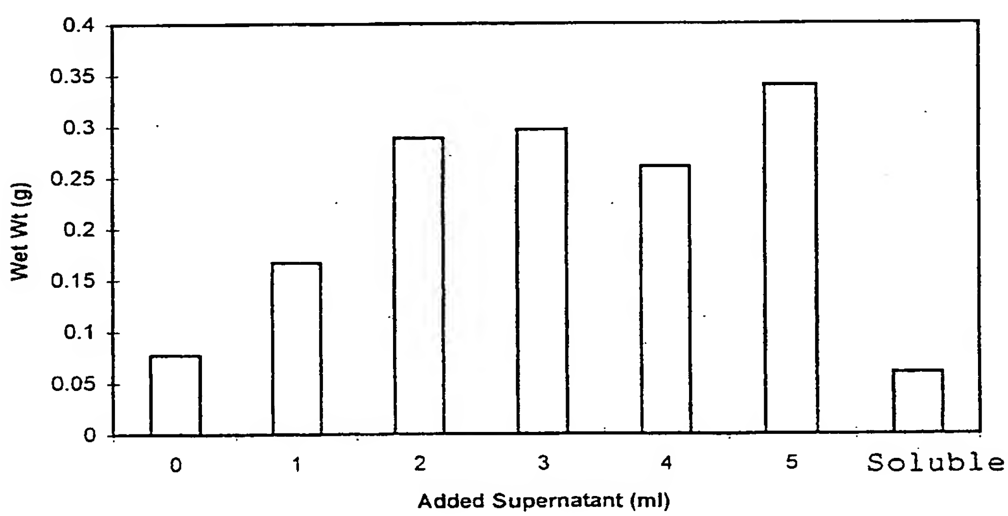


Figure 5

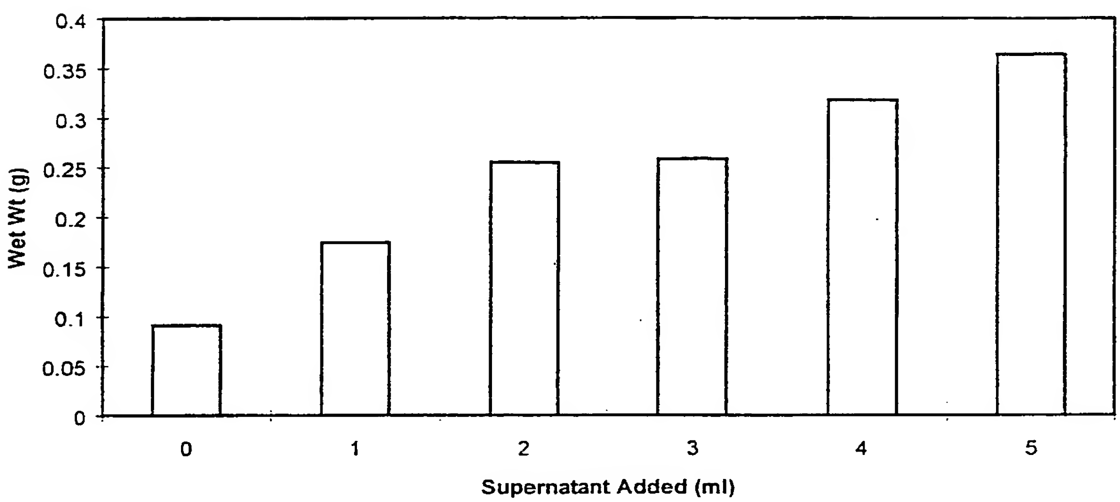



Figure 6

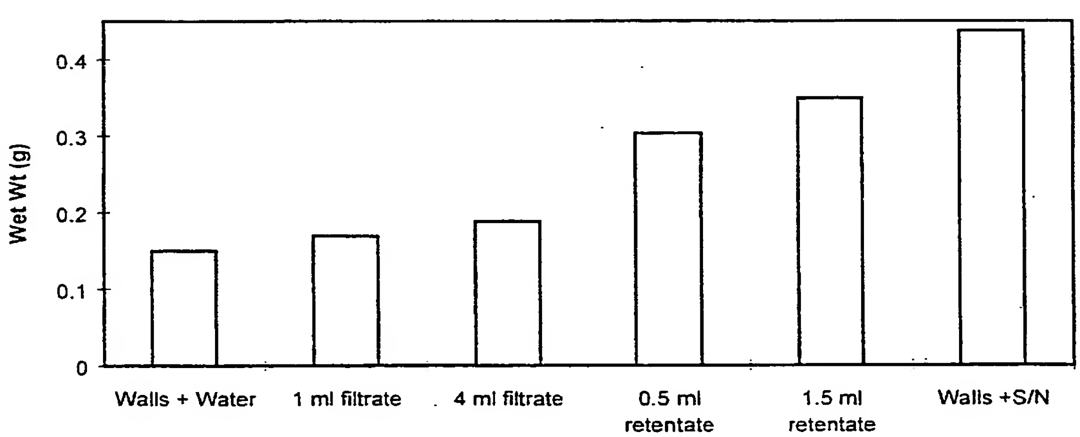


Figure 7

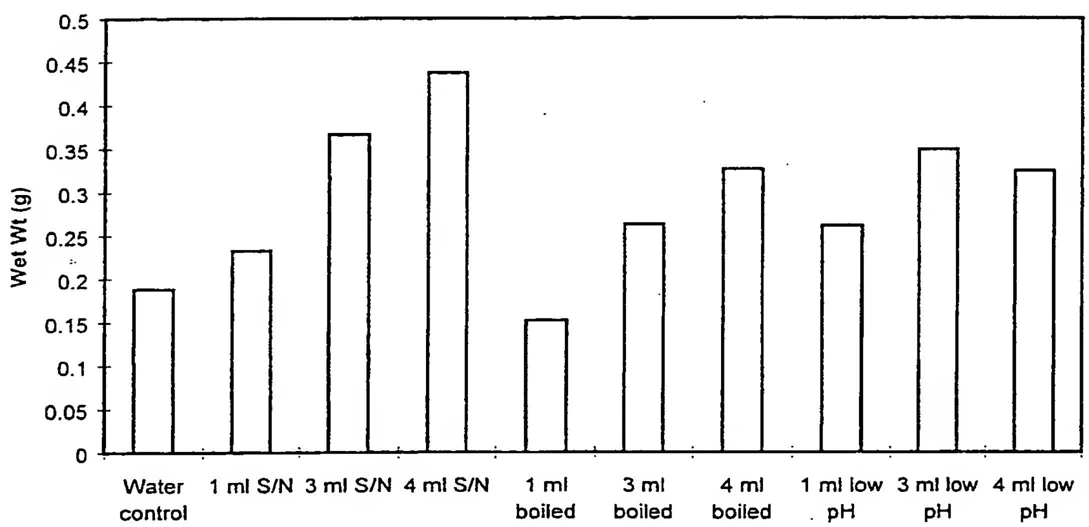



Figure 8

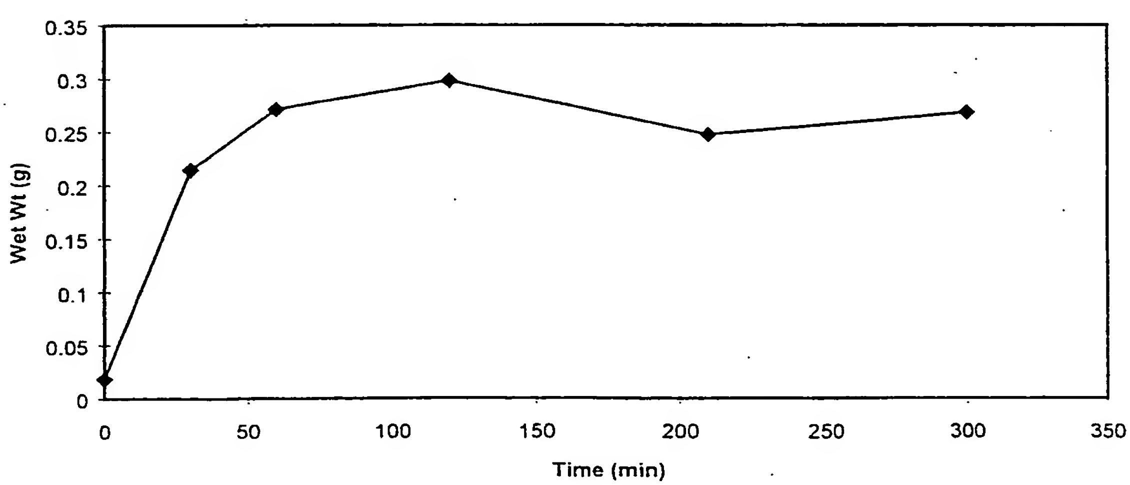


Figure 9

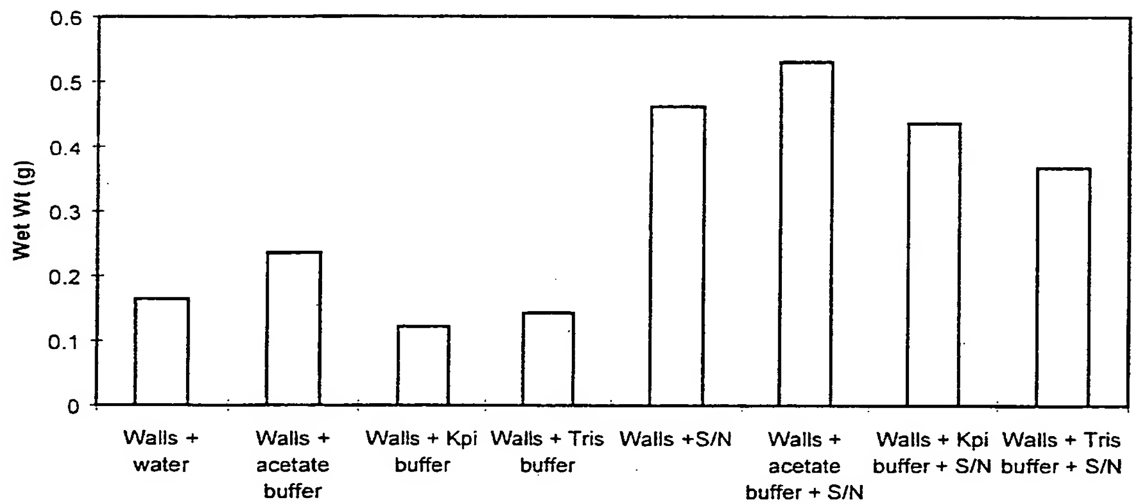
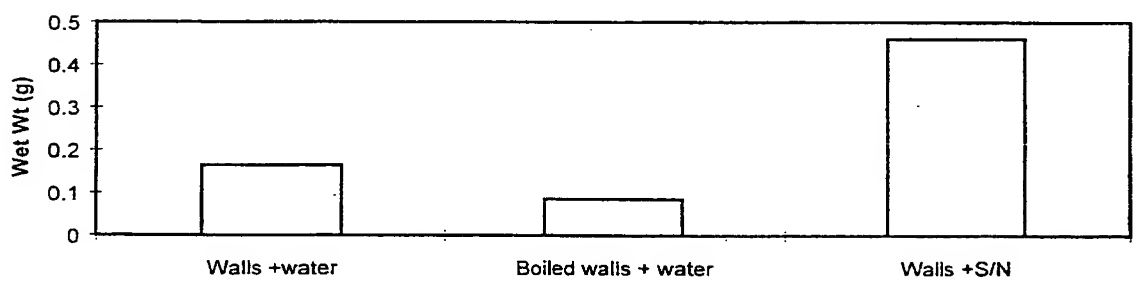



Figure 10



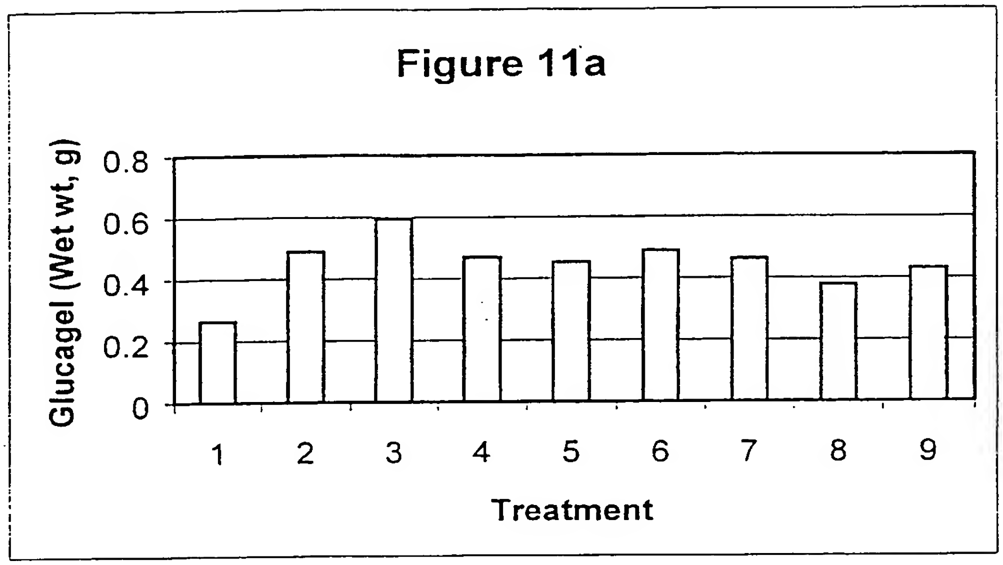


Figure 11a
Glucagel (Wet wt, g)
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
Treatment

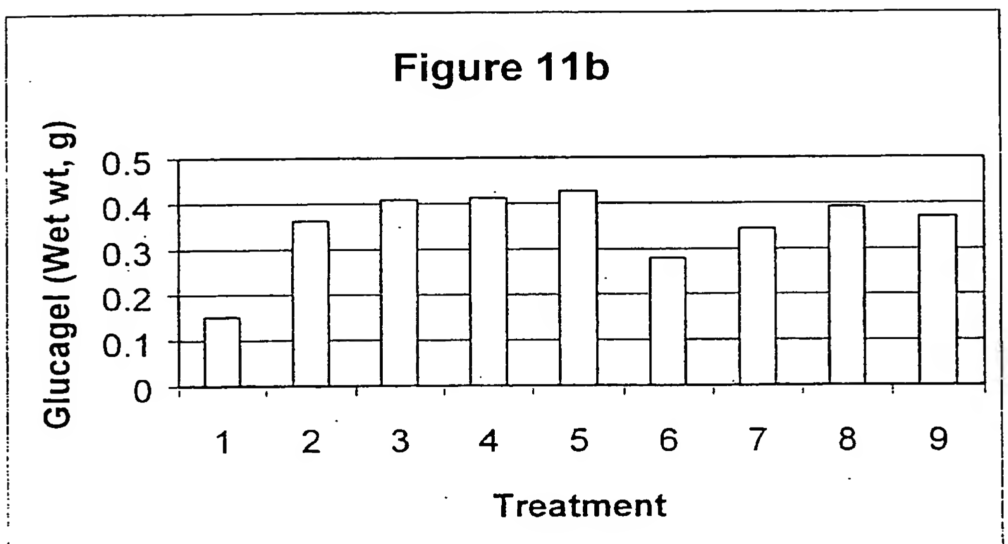


Figure 11b
Glucagel (Wet wt, g)
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
Treatment

Figure 12

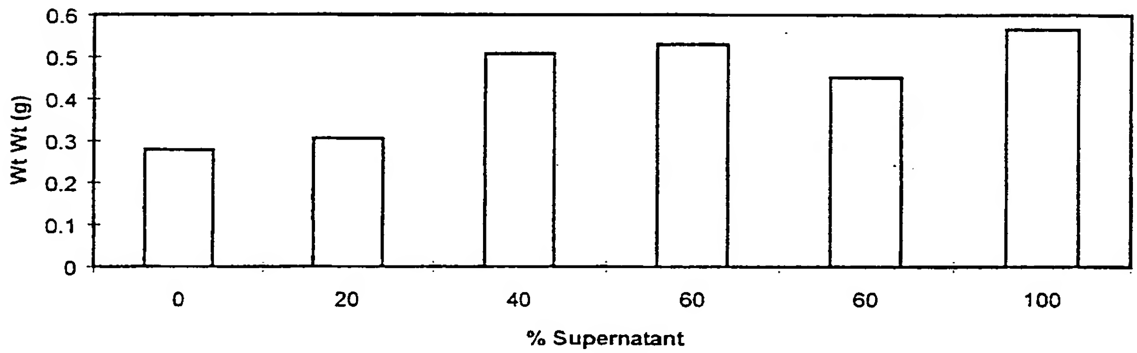



Figure 13

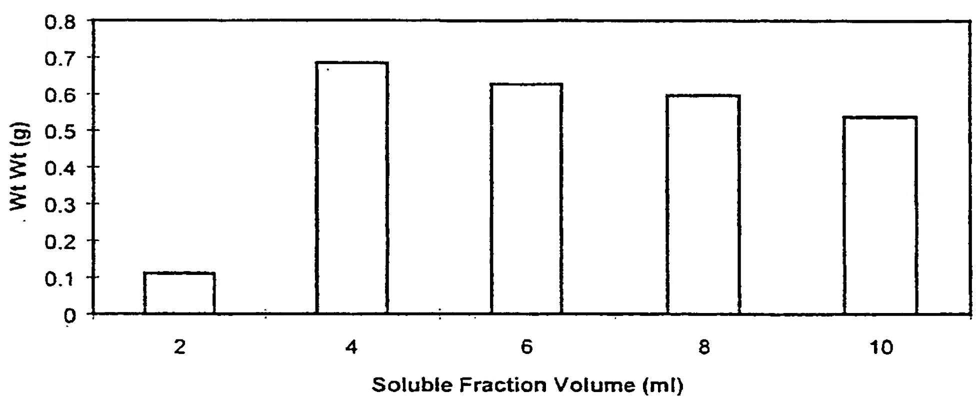


Figure 14

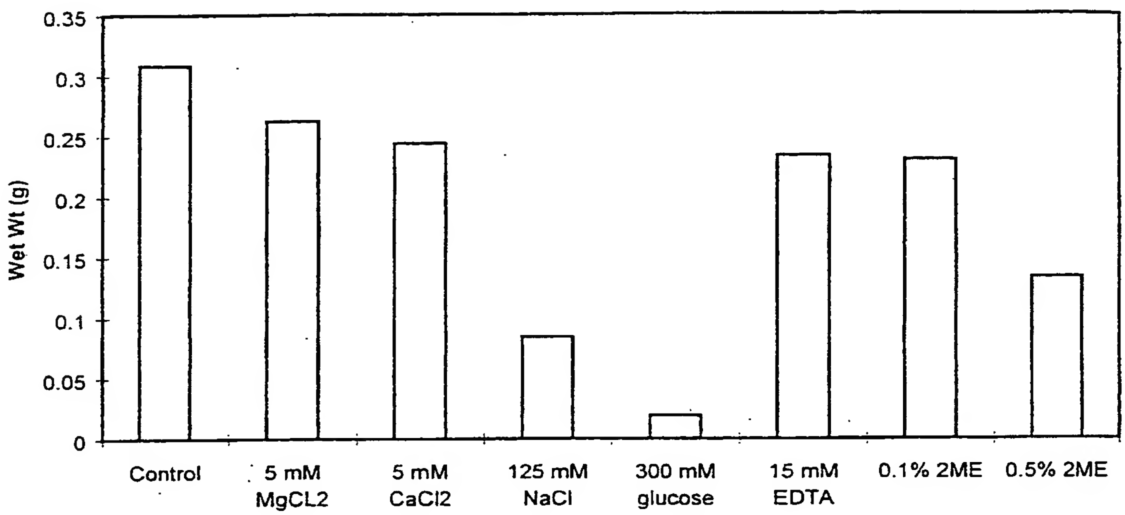

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Wet Wt (g)
Control
5 mM MgCL2
5 mM CaCl2
125 mM NaCl
300 mM glucose
15 mM EDTA
0.1% 2ME
0.5% 2ME

Figure 15

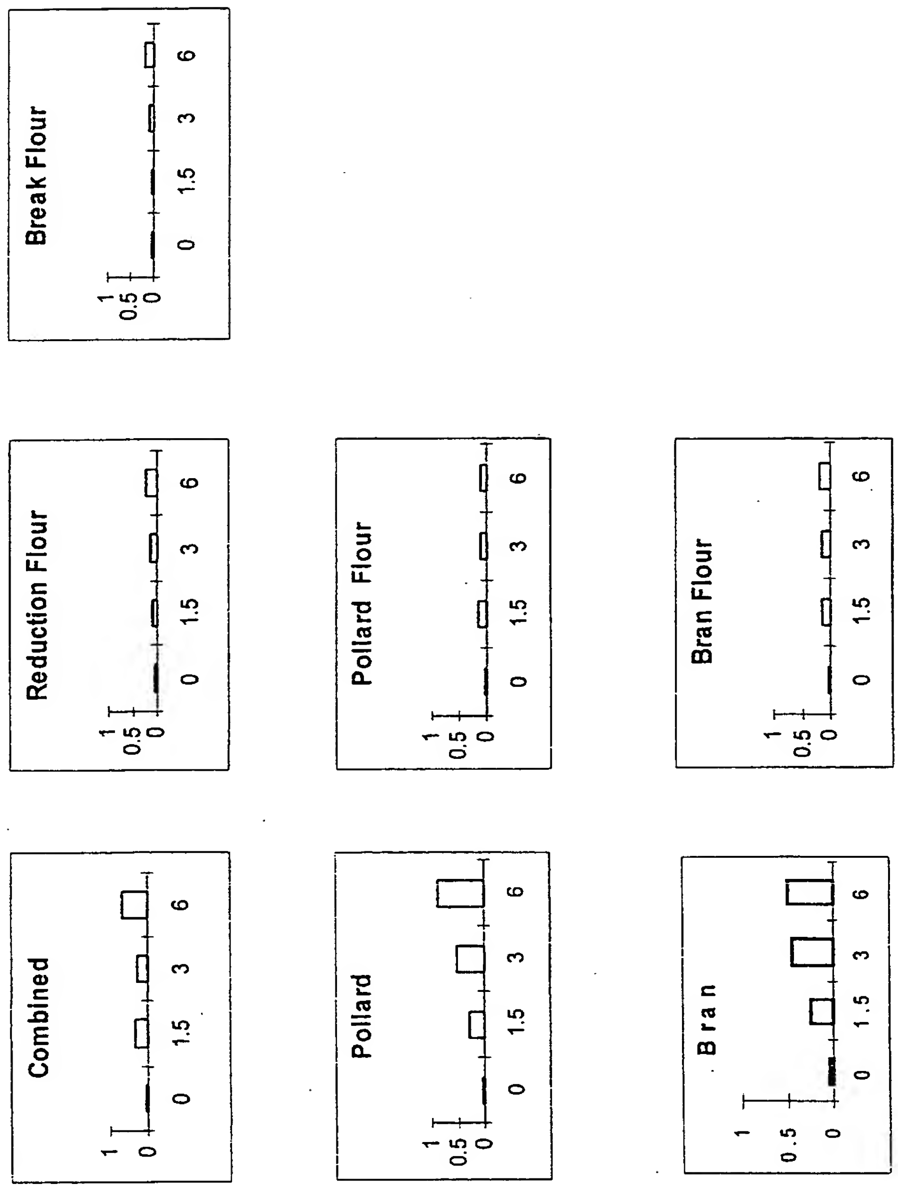
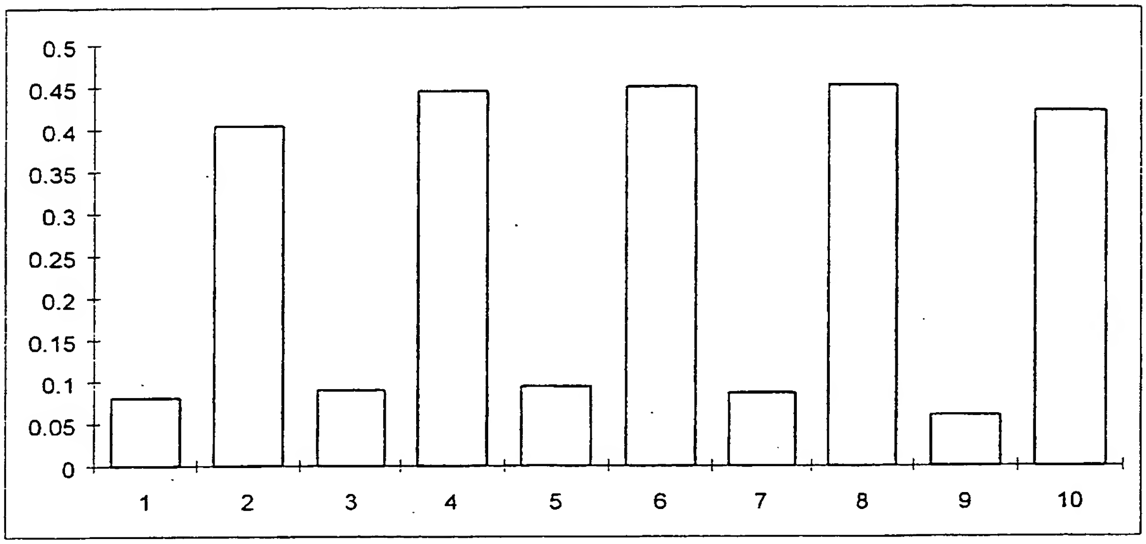

Break Flour
Reduction Flour
Pollard Flour
Bran Flour
Combined
Pollard
Bran
0
1.5
3
6
0.5
1

Figure 16


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
5
6
7
8
9
10

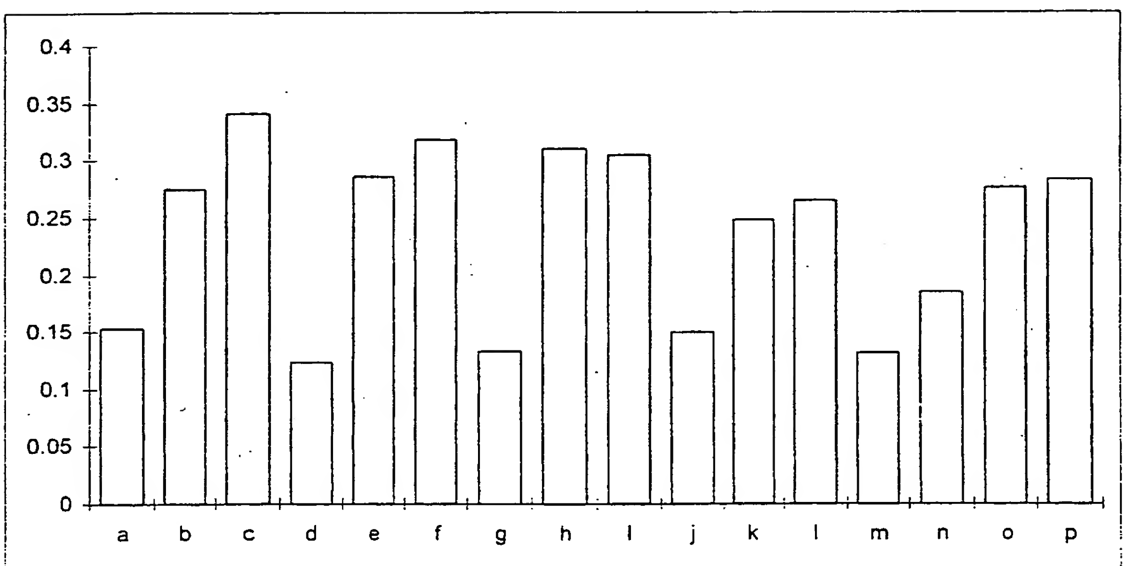

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
c
d
e
f
g
h
I
j
k
l
m
n
o
p

Figure 17

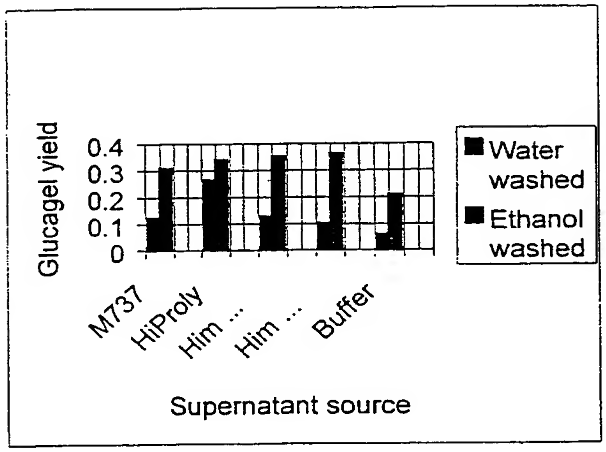



Figure 18

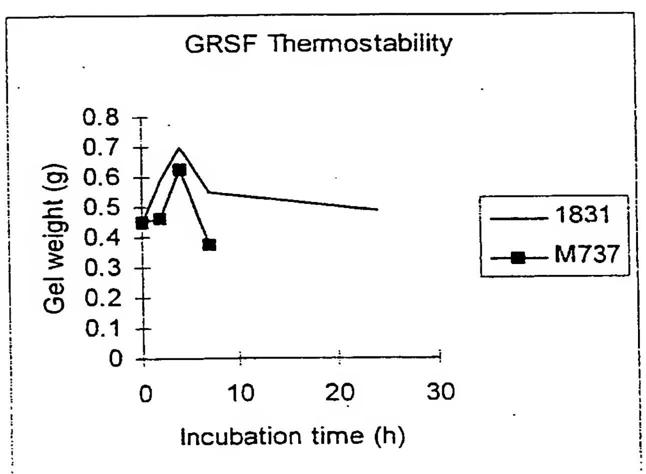
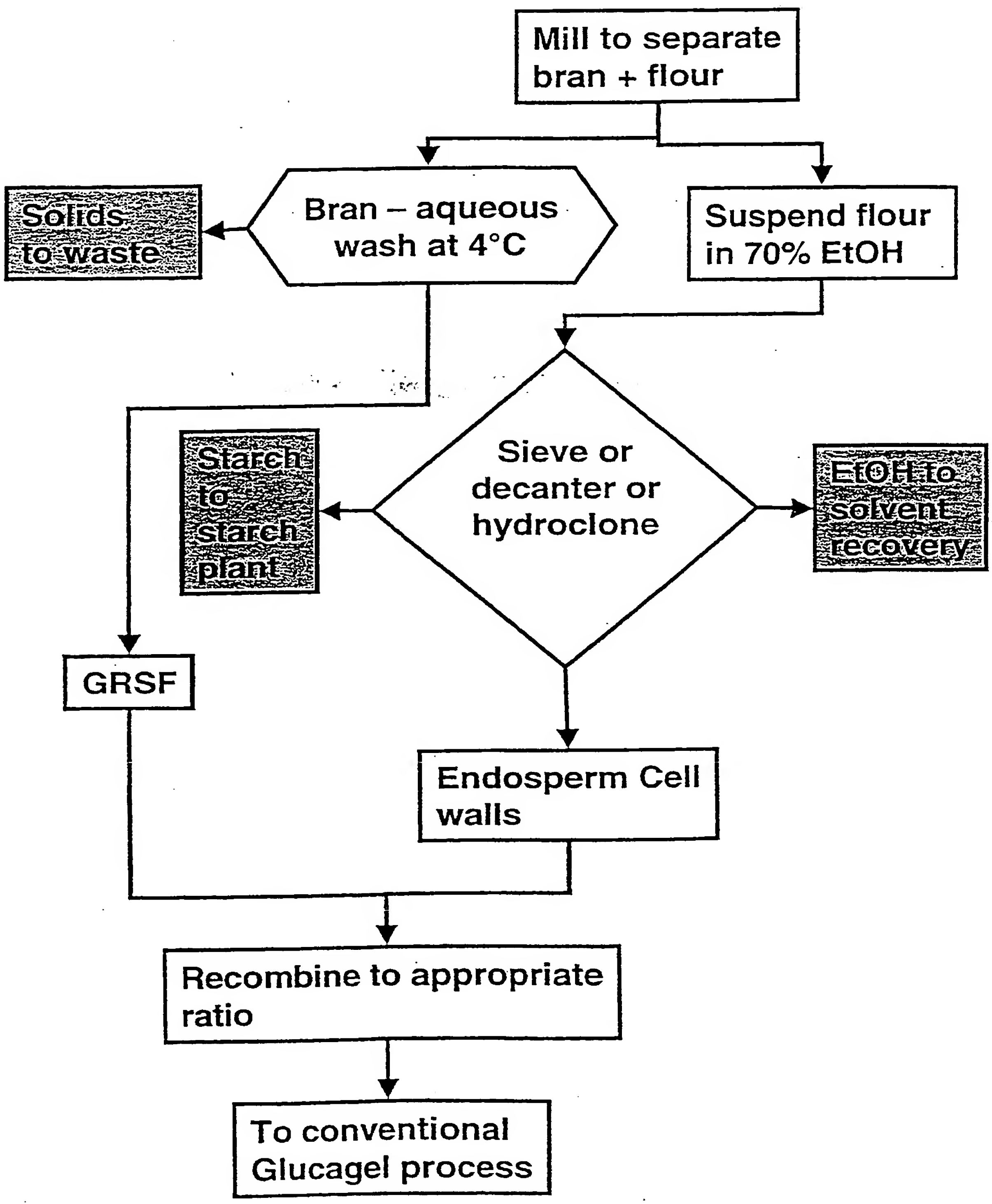


Figure 19


Mill to separate bran + flour
Bran – aqueous wash at 4°C
Solids to waste
Suspend flour in 70% EtOH
Sieve or decanter or hydroclone
Starch to starch plant
EtOH to solvent recovery
GRSF
Endosperm Cell walls
Recombine to appropriate ratio
To conventional Glucagel process

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(74) Agents: **HAWKINS, Michael, Howard** et al.; Baldwin Shelston Waters, NCR Building, 342 Lambton Quay, Wellington (NZ).

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WO 00/56268 A3

(54) Title: BETA GLUCAN EXTRACTION FROM CEREALS

(57) Abstract: The invention discloses a process for separating gelling β-glucan from cereals. The process involves: 1) extracting a soluble factor from cereal flour into solution, and not extracting β-glucans into solution; 2) separating the starch from the solid residue remaining after the first step; and 3) adding the extract from the first step to the non starch residue from the second step. The soluble factor causes gelling β-glucan to be released, from the non starch residue, into solution.

BNSDOCID: <WO 0056268A3 I >

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ00/00034

| A. | CLASSIFICATION OF SUBJECT MATTER |
|---|---|

Int. Cl. [7]: A23L 1/0522 1/0524 1/0534

According to International Patent Classification (IPC) or to both national classification and IPC

| B. | FIELDS SEARCHED |
|---|---|

Minimum documentation searched (classification system followed by classification symbols)
See electronic databases

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS & WPIDS: (cereal OR grain OR wheat OR barley OR oat? OR rye OR triticale OR corn OR rice) AND ((water OR aqueous)(3w)extract?) AND (remov? OR separat? OR extract?)

CAS, FSTA: (gel OR gelling) AND (water OR aqueous OR buffer OR solvent OR ethanol OR alcohol) AND (extract? OR remov? OR separat? OR refin?) AND (cereal OR grain OR wheat OR barley OR oat OR rye OR triticale OR corn OR rice OR millet OR sorghum) AND beta(w)glucan

WPIDS: (water OR aqueous OR buffer OR solvent OR ethanol OR alcohol) AND (extract? OR remov? OR separat? OR refin?) AND (cereal OR grain OR wheat OR barley OR oat OR rye OR triticale OR corn OR rice OR millet OR sorghum) AND beta(w)glucan

| C. | DOCUMENTS CONSIDERED TO BE RELEVANT |
|---|---|

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 5 106 640 (LEHTOMAKI ET AL) 21 April 1992<br>Whole Document | 1-43 |
| X | US 5 183 677 (LEHTOMAKI ET AL) 2 February 1993<br>Whole Document | 1-43 |
| X | US 4 028 468 (GARY A. Honer & ROY G. HYLDON) 7 June 1977<br>Whole Document | 1-43 |

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 June 2000 | 20 SEP 2000 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. (02) 6285 3929 | Authorized officer<br>J.H. CHAN<br>Telephone No : (02) 6283 2340 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 5 518 710 (R. BHATTY) 21 May 1996<br>Whole Document | 1-43 |
| X | EP 0 377 530 (ALKO LTD) 11 July 1990<br>Whole Document | 1-43 |

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ00/00034

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| US | 5518710 | NONE | | | | | |
| US | 5183677 | CA | 2007282 | EP | 377530 | FI | 890079 |
| | | JP | 2222658 | SU | 1812950 | US | 5106640 |
| US | 4028468 | CA | 1085384 | | | | |

END OF ANNEX



BNSDOCID: <WO________0056268A3_I_>